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(54)【発明の名称】　水不溶性徐放性組成物、その製剤及びその製造方法

(57)【要約】
【課題】　簡単でしかも高収率の製造方法により、G-CSFを沈殿化で安定化させるとともに徐放効果により生体内で数日間に亘り薬効を保持する水不溶性の粒子製剤を提供すること。
【解決手段】　亜鉛イオンと沈殿を形成する薬効を有する蛋白質あるいはペプチド、亜鉛イオンと沈殿を形成する主たる薬効をもたない蛋白質、亜鉛イオン、及び酸性ムコ多糖体とからなる。

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【特許請求の範囲】

【請求項１】 亜鉛イオンと沈殿を形成する薬効を有する蛋白質あるいはペプチド、亜鉛イオンと沈殿を形成する主たる薬効をもたない蛋白質、亜鉛イオン、及び酸性ムコ多糖体とからなる水不溶性徐放性組成物。

【請求項２】 前記薬効を有する蛋白質あるいはペプチドがG-CSF、IL-2、エタナセプトあるいは抗体であることを特徴とする請求項１記載の水不溶性徐放性組成物。

【請求項３】 薬効を有する蛋白質あるいはペプチドの水不溶性徐放性組成物中の含量が少なくとも0.01重量%であることを特徴とする請求項1記載の水不溶性徐放性組成物。

【請求項４】 前記主たる薬効を持たない蛋白質がヒト血清アルブミンあるいはγ-グロブリンであることを特徴とする請求項１記載の水不溶性徐放性組成物。

【請求項５】 前記主たる薬効を持たない蛋白質の水不溶性徐放性組成物中の含量が少なくとも1重量%であることを特徴とする請求項１記載の水不溶性徐放性組成物。

【請求項6】 前記亜鉛イオンの水不溶性徐放性組成物中の含量が少なくとも1重量%であることを特徴とする請求項１記載の水不溶性徐放性組成物。

【請求項7】 前記酸性ムコ多糖体がコンドロイチン硫酸、ヒアルロン酸、ヘパリン、ヘパラン硫酸、デルマタン硫酸あるいはケラタン硫酸及びそれらの塩の少なくともひとつであることを特徴とする請求項１記載の水不溶性徐放性組成物。

【請求項8】 前記酸性ムコ多糖体の水不溶性徐放性組成物中の含量が主たる薬効をもたない蛋白質含量の１/100以上あることを特徴とする請求項１記載の水不溶性徐放性組成物。

【請求項9】 請求項１記載の水不溶性徐放性組成物が凍結乾燥されたものであることを特徴とする水不溶性徐放性組成物。

【請求項10】 前記水不溶性徐放性組成物が皮下注射、皮内注射、及び筋肉内注射に適した形態であることを特徴とする請求項１から請求項9のいずれかに記載の水不溶性徐放性組成物。

【請求項11】 請求項１記載の組成物に、必要に応じて製剤学的に受容可能な添加物を加えたことからなることを特徴とする水不溶性徐放性製剤。

【請求項12】 前記製剤学的に受容可能な添加物が乳酸グリコール酸重合体、乳酸重合体、界面活性剤、防腐剤、又は安定化剤であることを特徴とする請求項11記載の製剤。

【請求項13】 請求項11記載の製剤が凍結乾燥されたものであることを特徴とする製剤。

【請求項14】 前記製剤が皮下注射、皮内注射、及び筋肉内注射に適した形態であることを特徴とする請求項11から請求項13のいずれかに記載の製剤。

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【請求項15】 亜鉛イオンと沈殿を形成する薬効を有する蛋白質あるいはペプチドの溶液、亜鉛イオンと沈殿を形成する主たる薬効をもたない蛋白質の溶液、亜鉛塩の溶液、酸性ムコ多糖体の溶液を混合して作製される水不溶性徐放性組成物の製造方法。

【請求項16】 （１）亜鉛イオンと沈殿を形成する薬効を有する蛋白質あるいはペプチドの溶液と亜鉛塩の溶液を混合し沈殿作成し、次いで（２）亜鉛イオンと沈殿を形成する主たる薬効をもたない蛋白質の溶液と酸性ムコ多糖体の溶液を混合することを特徴とする請求項１記載の水不溶性徐放性組成物の製造方法。

【請求項17】 請求項15又は請求項16記載の製造方法による水不溶性徐放性組成物を遠心分離により沈殿とし、その沈殿を亜鉛塩溶液で再懸濁することを特徴とする請求項１記載の水不溶性徐放性組成物の製造方法

【請求項18】 請求項17の遠心分離を少なくとも1,000 x g で行うことを特徴とする請求項１記載の水不溶性徐放性組成物の製造方法。

【請求項19】 前記亜鉛イオンと沈殿を形成する薬効を有する蛋白質あるいはペプチドの溶液、亜鉛イオンと沈殿を形成する主たる薬効をもたない蛋白質の溶液、亜鉛塩の溶液、酸性ムコ多糖体の溶液の混合をｐH4.5～8.5の条件で行うことを特徴とする請求項１記載の水不溶性徐放性組成物の製造方法。

【発明の詳細な説明】

【０００１】

【発明の属する技術分野】本発明は、亜鉛イオンと沈殿を形成する薬効を有する蛋白質あるいはペプチドと亜鉛イオンと沈殿を形成するが主たる薬効を持たない蛋白質を酸性ムコ多糖存在下において亜鉛塩の添加により共沈させた水不溶性徐放性組成物、その製剤及びその製造法、詳しくは亜鉛イオンと沈殿を形成するG-CSF等の蛋白質あるいはペプチド、亜鉛イオンと沈殿を形成するが主たる薬効を持たない蛋白質、酸性ムコ多糖体、及び亜鉛イオンからなる水不溶性沈殿を用いた水不溶性徐放性組成物、及びその製造法並びにそれを用いた徐放性製剤に関する。

【０００２】

【従来の技術】現在、好中球の減少が伴う疾患や症状に対して、G-CSF製剤が用いられている。その投与方法は静脈注射、皮下注射、点滴などであるが、投与が1日1回あるいは2回で連日投与となっている。これはG-CSFの血中安定性が悪く半減期が短いうえに薬効を維持するためにはある濃度以上のG-CSFが血中に存在する必要があるからである。そのために患者は連日投与という負担を強いられ、G-CSFの大量使用にもつながっている。従って、G-CSFの血中濃度を維持するための製剤化が必要とされている。

【０００３】現在、G-CSFのいくつかのアミノ酸を置換し、半減期を延ばした変異体の製剤も使用されている

が、半減期は2倍程度で投与回数の改善には至っていない。また、G-CSFのリジン残基のいくつかにポリエチレングリコール（PEG）を付加したPEG化G-CSFの開発もなされているが、PEG化による活性の低下や抗原性、生産コストなどを考慮するとさらに好ましいG-CSFの血中濃度維持製剤の開発が望まれている。

【０００４】薬物の徐放化は生体内で長時間薬物濃度を維持する良い手段であるが、蛋白質を用いる場合安定性を考えるとあまり過激な条件は使えず、その徐放製剤の作製には多くの制限を受ける。

【０００５】蛋白質の簡単な徐放化として穏やかな条件で沈殿をつくり用いるという方法があるが、蛋白質の種類により沈殿する条件が異なり、沈殿を形成する穏やかな条件を探すのは非常に困難をきわめる。さらに、その条件は高収率を約束するものでなければならない。蛋白質を沈殿させる方法として、等電点沈殿、塩析、金属イオンとの沈殿などが応用でき、実際インスリンでは亜鉛懸濁製剤が臨床で使われているが、生体内では沈殿の溶解が早く数日間効果を持続させるものではない。従って、数日薬効を維持させるには沈殿にした蛋白質をそのまま用いるのではなくさらに工夫を加えた徐放製剤が必要となってくる。

【０００６】あるいは、ゼラチンやヒアルロン酸などを用いたハイドロゲルをキャリアとする徐放剤も考えられるが、この場合、徐放効果を得るにはゲルと親和性をもつ塩基性あるいはヘパリン結合性の蛋白質には期待する効果は得られるが、G-CSFのように等電点が中性以下の蛋白質ではゲルに巻き込ませても自然拡散のために望ましい徐放効果は得られないと考えられる。

【０００７】又先行技術文献として本出願人が平成１３年６月１１日に出願した特願２００１－１７７５４８号があるが、この文献にはG-CSFについての記載がない。

【０００８】

【発明が解決しようとする課題】このように、G-CSFはもっとも臨床使用されているサイトカインのひとつでありながら数日間血中濃度を維持する製剤が開発されていないのが現状である。そこで、本発明は簡単でしかも高収率の製造方法により、G-CSFを沈殿化で安定化させるとともに徐放効果により生体内で数日間に亘り薬効を保持する水不溶性の粒子製剤を提供することを目的とするものである。

【０００９】

【課題を解決するための手段】本発明者らは、顆粒球コロニー刺激因子（G-CSF）が金属イオン、たとえばカルシウムイオン、で沈殿を形成することに注目し、その沈殿性粒子による徐放製剤の開発を試みた。前述したように蛋白質と多価金属イオンのみの沈殿は溶解し易く、そのままでは期待する徐放効果は得られないと予想され、実際、本発明者らが行った実験でも確認された。

【００１０】そこで、本発明者らは、G-CSFと金属イオンからなるこのような沈殿の溶解を抑える方法として、沈殿の組成物にさらに沈殿性の物質を加えることが有効でないかと考え、金属イオンを結合することが知られ薬効がほとんど無い蛋白質、例えばヒト血清アルブミンやγ－グロブリンなどをG-CSFの金属イオンとの沈殿に含有させることを試みた。この場合、G-CSFとこれらの蛋白質とを共に沈殿できる多価金属イオンを選択することが重要である。実際ヒト血清アルブミンとγ－グロブリンはカルシウムイオンでは沈殿を形成しないことから、本発明者らはいくつかの多価金属イオン、具体的には、カルシウム、亜鉛、銅、鉄、アルミニウム、錫、ニッケルの各イオンについて沈殿形成を調べた。結果として、カルシウム、鉄、アルミニウム、ニッケルなどではまったく沈殿を起こさず、亜鉛イオンがもっとも望ましいことが分かった。使用する塩としては酢酸亜鉛あるいは塩化亜鉛が望ましく、また、沈殿の収率をあげるためには亜鉛の濃度は５ｍＭ以上が望ましいことがわかった。5mM以上の濃度の亜鉛イオンによりG-CSFは95%以上沈殿させることができた。

【００１１】さらに、本発明においては酸性ムコ多糖体を添加することにより、上記混合蛋白質をさらに効率よく沈殿でき、徐放性もさらに向上することを見出した。ムコ多糖体とはコンドロイチン硫酸、ヒアルロン酸、ヘパリン、ヘパラン硫酸、デルマタン硫酸、ケラタン硫酸およびこれらの塩などがあるが、コンドロイチン硫酸およびその塩が最も望ましい。

【００１２】たとえば、20 mg/mlのγ-グロブリンでは10mMの塩化亜鉛で80～90%ほど沈殿するが、コンドロイチン硫酸を加えることにより99%が沈殿することを確認した。また、10mg/mlのヒト血清アルブミンでは20mMの塩化亜鉛では沈殿は10%程度であるが、コンドロイチン硫酸を加えることにより95%以上沈殿させることができた。

【００１３】なお、前記文献（特願2001-177548号）には生物学的活性を有する蛋白質又はペプチド等に必要に応じて血清蛋白質を添加することが記載されているが、本発明ではG-CSF、後述のIL-2、エタナセプト又は抗体と亜鉛イオンの沈殿に必ず亜鉛イオンと沈殿を形成する主たる薬効をもたない蛋白質を含有させることが前述の如く徐放効果を高めた沈殿を作製するのに不可欠なものである。

【００１４】このように、本発明者らはG-CSFの亜鉛イオンによる沈殿を含む溶液にさらに、γ-グロブリンやヒト血清アルブミンなどと酸性ムコ多糖体を加え、新たな沈殿を形成させる方法においては、それらの相互作用により沈殿形成が促進され99%以上G-CSFを沈殿させることができることを見出し、本発明に至った。この沈殿は注射針を通るのに十分な細かさであり、注射剤として使用可能である。

【００１５】さらに、酸性ムコ多糖体を加えたγ-グロ

ブリンやヒト血清アルブミン沈殿形成の効果は、G-CSFの亜鉛イオンによる沈殿形成の収率を高めるだけでなく生体内での徐放効果も非常に高まることがマウスを使った実験において確認され本発明を完成した。

【００１６】本発明はｐH4.5～8.5の間で沈殿を形成させることを特徴とする。すなわち、中性に近いpHであるためG-CSFのように極端なｐHで活性を失うような蛋白質においてもその活性を損なわずに製造が可能である。さらに、沈殿にG-CSFを高い割合で含ませることを特徴とする。最適な条件では99%以上である。

【００１７】本発明の水不溶性徐放性組成物は各構成成分を加える順番を気にせずに沈殿を形成させることも可能であるが、高収率でG-CSFを沈殿に含ませるには、10～1000μg/mlのG-CSF溶液を5mM以上の最終濃度の酢酸亜鉛あるいは塩化亜鉛でまずG-CSFを沈殿させることが望ましい。次に、最終濃度が0.5～20 mg/mlになるようヒト血清アルブミンとアルブミンに対して１/2～1/100の最終濃度になるようにコンドロイチン硫酸を加えることで本発明の水不溶性徐放性組成物を作ることが望ましい。

【００１８】さらに、遠心操作を行うことにより沈殿を固めた後、亜鉛イオンを含む水溶液で再懸濁して用いることもできる。さらに、懸濁液にマンニトールなどを加え凍結乾燥したものを注射用水で再懸濁し用いることができる。沈殿はEDTAに溶かし、非変性条件の電気泳動をおこない、G-CSFが沈殿作成中に変性をおこしていないことが確認された。また、凍結乾燥した製剤では37℃で1か月以上G-CSFは変性していないことが確認された。本発明のG-CSF製剤は皮下注射あるいは筋肉注射で投与が可能である。本発明のG-CSF製剤は、マウスにおいては一回の投与で10日以上その薬効を維持するだけでなく、その投与量はその薬効を維持するために連日投与で使用されるG－CSF量の1/10以下で充分であった。また、本発明のG-CSF製剤は、ラットにおいては一回の投与で6日以上その薬効を維持し、その時の投与量はその薬効を維持するために連日投与で使用されるG-CSF量の1/3以下で充分であった。

【００１９】本発明の製造方法による徐放製剤はG-CSFのみならず、亜鉛イオンと沈殿を作る薬効を持つ蛋白質あるいはペプチドに応用可能である。例えば、ヒルジン、インターロイキン-2（IL-2）、エタナセプト、モノクローナル抗体、γ-カルボキシグルタミン酸を持つ蛋白質、His-タグをつけた組み換え体蛋白質製剤などが含まれる。特に微量で薬効を示す因子は望ましい。

【００２０】例えば、モノクローナル抗体を本発明の方

法により徐放製剤化したものはin vitroの実験においてG-CSFと同様な徐放効果を示した。また、IL-2は塩化亜鉛で沈殿するが、ヒト血清アルブミンとコンドロイチン硫酸を添加して沈殿を形成させたほうがより沈殿に含まれることが確認された。

【００２１】

【実施例】以下に本発明の実施例について説明する。

（試験例１）G-CSFの各種金属イオンでの沈殿作成の試み

150μg/ml G-CSF（中外製薬）溶液500μlに500mMの金属塩（塩化錫、塩化アルミニウム、塩化銅、塩化鉄、塩化亜鉛、塩化カルシウム、塩化ニッケル）20μlを加え撹拌後静置して目視により沈殿形成を確認した。６種類すべての金属塩に対して沈殿を形成した。

【００２２】（試験例２）ヒトγ-グロブリンおよびヒト血清アルブミンの各種金属イオン形成の試みとムコ多糖体が沈殿形成に与える効果

10mg/mlヒトγ-グロブリン（シグマ社）あるいは10mg/mlヒト血清アルブミン（シグマ社）溶液500μlに500mMの金属塩（塩化錫、塩化アルミニウム、塩化銅、塩化鉄、塩化亜鉛、塩化カルシウム、塩化ニッケル）20μlを加え撹拌後静置して目視により沈殿形成を確認した。両蛋白質とも塩化錫および塩化亜鉛の添加により白濁した。これらの溶液にさらに20mg/mlのコンドロイチン硫酸を添加したところ、塩化カルシウム添加溶液以外のすべての溶液で沈殿が確認された。塩化錫および塩化亜鉛に関してはさらに強く白濁した。塩化アルミニウム、塩化鉄、塩化ニッケル添加溶液の沈殿は弱くしかも塩化アルミニウムと塩化鉄に関しては均一な沈殿ではなかった。結果からγ-グロブリンやヒト血清アルブミンを沈殿させる金属イオンとしては亜鉛が最適であることが分かった。

【００２３】（試験例３）γ-グロブリンの塩化亜鉛とコンドロイチン硫酸による沈殿作成

20mg/mlのγ-グロブリン（シグマ社）500μlに0.5M塩化亜鉛（ｐH5.4）をそれぞれ20、10、5、2、1、0.5μl（20～0.5mM）加えたものと20mg/mlのγ-グロブリン500μlに0.5M塩化亜鉛10μlと20 mg/mlコンドロイチン硫酸160μlを加えたものを撹拌後5分間室温に放置した。次に約10,000 x gで10分間遠心分離し、上清のγ-グロブリン濃度をProtein Assay試薬（バイオラッド社）を用いた発色法により測定した。

【００２４】表１に示すようにγ-グロブリンは塩化亜鉛濃度が高くなるにつれ沈殿量が多くなる。また、コンドロイチン硫酸は沈殿形成を促進している。

表１γ-グロブリンと亜鉛イオンの沈殿形成における亜鉛塩の濃度の影響とコンドロイチン硫酸（CS）の効果

塩化亜鉛濃度（mM）	0.5	1	2	5	10	20	10 +CS
上清に残るγ-グロブリンの割合（%）	39	37	37	30	13	5	2

【００２５】（試験例４）γ-グロブリンの塩化亜鉛とヒアルロン酸による沈殿作成
20mg/mlγ-グロブリン300μlにミリQ水あるいは10mg/mlヒアルロン酸200μlを混ぜ、500μlとした溶液に0.5M塩化亜鉛（pH5.4）を20、10、５、２、１μl（20～1mM）加え撹拌後室温に5分間放置した。次に約10,000xgで＊10＊４℃、30分間遠心分離後、上清のγ-グロブリン濃度をProtein Assay試薬（バイオラッド社）を用いた発色法により測定した。表２に示すようにコンドロイチン硫酸同様ヒアルロン酸も塩化亜鉛によるγ-グロブリンの沈殿形成を促進した。

表２γ-グロブリンと亜鉛イオンの沈殿形成における亜鉛塩の濃度の影響とコンドロイチン硫酸（CS）の効果

亜鉛濃度（mM）	ヒアルロン酸なし					ヒアルロン酸添加				
	1	2	5	10	20	1	2	5	10	20
上清に残るγ-グロブリンの割合（%）	70	76	47	15	5	41	11	4	3	3

【００２６】（試験例５）γ-グロブリン・亜鉛・コンドロイチン硫酸沈殿におけるγ-グロブリンとコンドロイチン硫酸の濃度比
20mg/mlγ-グロブリン（シグマ社）200μlに20mg/mlコンドロイチン硫酸（和光純薬）を100μl（２：１）、50μl（４：１）、25μl（８：１）、12.5μl（16：1）あるいは10mg/mlコンドロイチン硫酸を40μl（10：１）、20μl（20：１）、８μl（50：１）、４μl（100：１）あるいは1mg/mlコンドロイチン硫酸を８μl（500：１）、４μl（1000：１）を混合し、ミリQ水で400μlとした溶液に0.5M塩化亜鉛（pH5.4）を8μl加え室温で5分間放置した。次に約10,000xg、4℃で10分間遠心分離し、上清のγ-グロブリン濃度をProtein Assay試薬（バイオラッド社）を用いた発色法により測定した。結果はγ-グロブリンとコンドロイチン硫酸の比が2～100：１においては90%以上が沈殿していた。また最も沈殿していたのは比が8～20：１の時であった。

【００２７】（試験例６）ヒト血清アルブミンの酢酸亜鉛とコンドロイチン硫酸との沈殿作成
50 mg/mlヒト血清アルブミン（シグマ社）100μlに0.5M酢酸亜鉛（pH6.0）を用いて亜鉛濃度が1、５、10、15、20mMとなるように加え、ミリQ水で500μlとし撹拌後37℃で10分間放置した。白濁したので約10,000x gで室温10分間遠心分離し、上清のヒト血清アルブミンの濃度をProtein Assay試薬（バイオラッド社）を用いた発色法により測定した。すべての亜鉛濃度で沈殿したヒト血清アルブミンの割合は2～10%のみであった。次に、50mg/mlヒト血清アルブミン（シグマ社）100μlに0.5M酢酸亜鉛（pH6.0）を用いて亜鉛濃度が1、５、10、20、40mMとなるように加え、それぞれの亜鉛濃度でコンドロイチン硫酸の濃度が0.5、1、2.5、5、10mg/mlになるように20mg/mlコンドロイチン硫酸とミリQを用いて500μlの溶液に調製し撹拌後37℃で10分間放置した。沈殿が形成されたので約10,000xg室温で10分間遠心分離後、上清のアルブミン濃度をProtein Assay試薬（バイオラッド社）を用いた発色法により測定した。表３に示すように亜鉛濃度が20mM、コンドロイチン硫酸量が1mg/ml、つまりヒト血清アルブミン：コンドロイチン硫酸が10：１の濃度で最もよく沈殿した。

表３　10mg/mlのヒト血清アルブミン（HSA）の沈殿量に与える亜鉛塩とコンドロイチン硫酸の濃度の影響

		亜鉛濃度　(mM)				
		1	5	10	20	40
コンドロイチン硫酸濃度（mg/mL）		上清に残るHSAの割合（%）				
	0.5	83	15	4	1	5
	1	80	29	2	1	1
	2.5	85	56	14	3	1
	5	77	84	37	9	3
	10	78	84	71	27	7

【００２８】（実施例1）G-CSFのヒト血清アルブミン、酢酸亜鉛、コンドロイチン硫酸混合物による沈殿徐放製剤の作成とG-CSFのin vitroでの溶出試験

G-CSF(150μg/ml)20μl、酢酸亜鉛（0.5M）12μl、ミリQ水103μlを混ぜ懸濁させ、さらにヒト血清アルブミン(20mg/ml)150μl、コンドロイチン硫酸（20mg/ml）15μlを加え撹拌後、37℃で10分間静置した。約10,000x g、室温で10分間遠心後、上清をすて、150μlの20mM酢酸亜鉛、0.5Mカルボキシメチルセルロース、5%マンニトールで再懸濁し徐放製剤とした。またコントロールは20μl G-CSF(150μl/ml)をミリQ水で150μl溶液とした。沈殿からのG-CSFの溶出試験は24ウェル-セルカルチャーインサートシステム（ファルコン社）を用いておこなった。3μmのポアサイズの膜を持つインサートに100μlの試料を入れた後、インサートを900μlの0.3%BSA PBSの入ったウェルにいれ、シェーカーで揺らした。1時間毎にインサートを新しいウェルに移し変え、インサートの膜を通してウェルに溶出してきたG-CSFをELISAで定量し、溶出量を1時間毎に8時間まで積算した。図1に示すように8時間後の沈殿からの積算溶出量は約５%程度でかなり徐放効果があることが示された。

【００２９】（実施例2）G-CSF徐放製剤投与正常マウスにおけるG-CSFの血中動態と薬効

167μlの150μg/ml G-CSF（中外製薬）に20μlの0.5M酢酸亜鉛と25μlの20mg/mlのコンドロイチン硫酸を加えた後50mg/mlヒト血清アルブミン100μlと注射用水188μlを加え撹拌し37℃で5分間放置した。約10,000x gで10分間遠心分離後、上清を捨て、沈殿を250μlの20mM酢酸亜鉛、0.5%カルボキシメチルセルロース含有５%マンニトール溶液に懸濁した。この懸濁液100μlを体重約25gの正常マウス（C3H）3匹に筋肉注射した。コントロールとして33μlの150μg/ml G-CSFに50mg/mlヒト血清アルブミン100μlと注射用水117μlを加え、５%マンニトールとした溶液の100μlを体重約25gの正常マウス（C3H）3匹に筋肉注射した。投与前、および投与後4時間、1、２、3、4、5、6、7、8、9、10、11、14日に採血を行い、G-CSFの血中動態は6日までELISAで測定し、薬効として14日まで血球計算盤にて白血球数を測定した。図2に示すようにG-CSFそのものを投与したものは4時間後ではかなり血中に存在しているが、24時間後は検出限界以下であった。それに比べ、徐放製剤の方は6日後においても数100pg/mlの濃度で血中に存在していた。薬効に関しては11日間白血球を増加させる効果を維持していた。

【００３０】（実施例3）凍結乾燥G-CSF徐放製剤の作成とその薬効および安定性

G-CSF(150μg/ml)1.334ml、酢酸亜鉛（0.5M）0.8ml、注射用水12.866mlを混ぜ撹拌後、20mg/mlコンドロイチン硫酸1mlと50mg/mlヒト血清アルブミンを4ml加え撹拌し、10分間37℃で放置した。約10,000x gで10分間遠心後、上清をすて、18mlの20mM酢酸亜鉛で再懸濁し、１gのマンニトールを添加し、撹拌した。撹拌しながら1.2mlずつバイアルに分注し、凍結乾燥した。２週間37℃においた凍結乾燥製剤を0.5%カルボキシメチルセルロースあるいは注射用水1mlで懸濁させた溶液300μlを約25gのマウスに皮下注射した。投与前、および投与後1、２、3、4、5、7、8、9、10、11日に採血を行い、血球計算盤にて白血球数を測定した。図3に示すように再凍結乾燥製剤において10日間薬効が観察された。バイアルに凍結乾燥したG-CSFを37℃で1か月保管し、１mlのEDTA（100mM）で沈殿を溶かし10μlをマルチゲル2/15（第１化学）を用いネイティブ電気泳動し、銀染色およびウェスターンブロティング後抗G-CSF抗体でG-CSFを検出することによりG-CSFの変性、分解、凝集などを調べた。その結果、製剤のG-CSFの電気泳動における移動度はまったく変わらず、分解や凝集に由来するようなバン

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ドは認められなかった。結果からG-CSF凍結乾燥製剤は非常に安定であることがわかった。

【００３１】（実施例4）G-CSF徐放製剤投与正常ラットにおけるG-CSFの薬効

32μlの780μg/ml G-CSF（中外製薬）に20μlの0.5M酢酸亜鉛と25μlの20 mg/mlコンドロイチン硫酸を加えた後50 mg/mlヒト血清アルブミンを100μlと注射用水323μlを加え撹拌し37℃で5分間放置した。約10,000ｘｇで10分間遠心後、上清を捨て、沈殿を500μlの20mM酢酸亜鉛、0.5%カルボキシメチルセルロース含有５%マンニトール溶液に懸濁した。この懸濁液200μlを体重約120gの正常ラット（ウィスター）2匹に皮下注射した。対照としてG-CSFを含まない沈殿懸濁液（vehicle）を単回と25μg/ml G-CSF，10mg/mlヒト血清アルブミン含有5%マンニトール液200μlを連日5日、体重約120ｇの正常ラット（ウィスター）２匹ずつに皮下注射した。投与前、および投与後1、２、3、4、6、7、8日に採血を行い、薬効として8日まで血球計算盤にて白血球数を測定した。図４に示すように対照の連日投与のものは投与を止めると白血球数はもとに戻るが、徐放製剤は1回の投与で6日間白血球を増加させる効果を維持していた。

【００３２】(実施例5)抗TNF抗体の亜鉛イオン、ヒト血清アルブミンコンドロイチン硫酸混合沈殿からの抗TNF抗体のin vitroでの溶出

抗TNF抗体（10mg/ml）25μl、塩化亜鉛（0.5M）20μl、ミリQ水180μlを混ぜ、撹拌し、さらにヒト血清アルブミン（20mg/ml）250μl、コンドロイチン硫酸（20mg/ml）25μlを加え撹拌し、10分間37℃で静置した。約10,000ｘｇ、室温で10分間遠心分離後、上清をすて、500μlの20mM塩化亜鉛含有0.5Mカルボキシメチルセルロースで再懸濁し徐放製剤とした。またコントロールは10μl抗TNF(10mg/ml)と180μlヒト血清アルブミン(20mg/ml)を加えた溶液とした。沈殿からの抗TNF抗体の溶出試験は24ウェル-セルカルチャーインサートシステム（ファルコン社）を用いておこなった。3μmのポアサイズの膜を持つインサートに100μlの試料をいれた後、インサートを900μlの0.3%BSA、PBSの入ったウェルにいれ、シェーカーで揺らした。1時間毎にインサートを新しい＊

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＊ウェルに移し変え、インサートの膜を通してウェルに出してきた抗TNF抗体をELISAで定量し、溶出量を1時間毎に8時間まで積算した。図５に示すように8時間後の沈殿からの溶出は10%以下でかなり徐放効果があることが示された。

【００３３】(実施例6)IL-2の塩化亜鉛による沈殿形成およびヒト血清アルブミンとコンドロイチン硫酸を含む沈殿の形成

IL-2（100μg/ml）50μl、塩化亜鉛（0.5M）4μl、ミリQ水46μlを混ぜ撹拌し10分間室温に放置した。約10,000ｘｇで室温10分間遠心分離後、上清を回収、沈殿は100μlの50mM EDTAで溶かした。また、IL-2（100μg/ml）50μl、塩化亜鉛（0.5M）4μlを混ぜ撹拌後、コンドロイチン硫酸（20mg/ml）5μl、ヒト血清アルブミン（50mg/ml）20μl、ミリQ水21μlを混ぜ再度撹拌し10分間放置した。約10,000ｘｇで10分間遠心後、上清を回収、沈殿は100μlの50mMEDTAで溶かした。これらの上清及び沈殿を溶かした液10μlを10μlの電気泳動サンプル処理液と混ぜ、マルチゲル10/20（第１化学）を用いSDS電気泳動をおこなった。クマシーブリリアントブルー染色を行ない、IL-2を検出した。結果は、どちらの条件においてもIL-2は沈殿の方に検出され、上清の方には検出されなかった。このことはIL-2もG-CSF同様の方法で徐放製剤化が可能であることを示している。

【図面の簡単な説明】

【図1】実施例７の製剤からG-CSFの溶出をin vitroの実験でG-CSFの溶液と比べて示した図である。

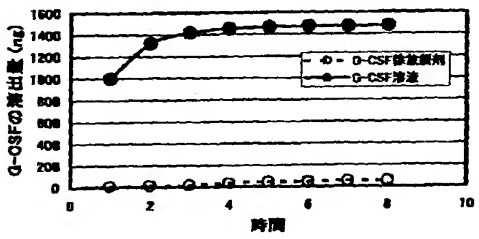
【図2】実施例８の製剤をマウスに筋肉内注射後G-CSFの血中濃度の推移とマウスの白血球数の変化を示す図である。

【図3】実施例9の凍結乾燥製剤をマウスに皮下投与した時、G-CSFの徐放により薬効が維持されていることを示す図である。

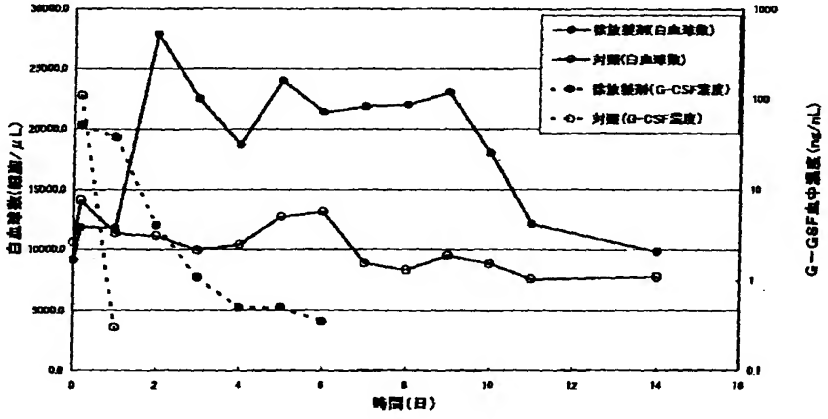
【図4】実施例10の製剤をラットの皮下に注射し、対照としてG-CSF溶液の連日投与との薬効の比較を示す図である。

【図5】実施例７の製剤から抗TNF抗体の溶出をin vitroの実験で抗TNF抗体の溶液と比べて示した図である。

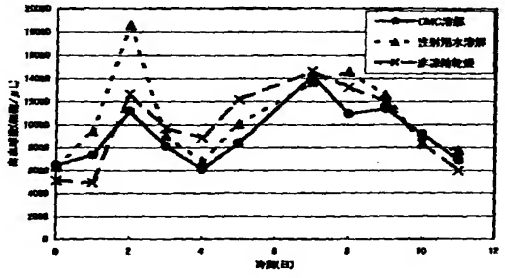
【図１】



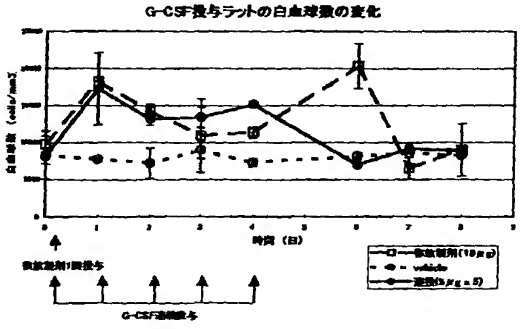
【図２】



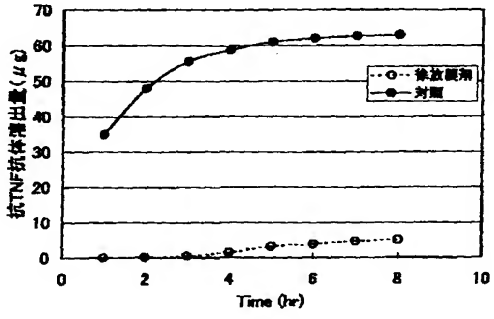
【図３】



【図４】



【図５】



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(54) WATER-INSOLUBLE SUSTAINED-RELEASE COMPOSITION, ITS PREPARATION AND METHOD FOR PRODUCING THE SAME

(57)Abstract:
PROBLEM TO BE SOLVED: To provide a water-insoluble particulate preparation easily producible in high yield, effective for stabilizing G-CSF by precipitation and keeping the drug effect for several days in vivo by the sustained release effect.
SOLUTION: The particulate preparation is composed of a protein or peptide having medicinal action and forming a precipitate with zinc ion, a protein free from remarkable medicinal action and forming a precipitate with zinc ion, zinc ion and an acidic mucopolysaccharide.

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* NOTICES *

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CLAIMS

[Claim(s)]
[Claim 1] The protein which has the drug effect which forms zinc ion and precipitate or a peptide, zinc ion, protein without the main drug effect which forms precipitate and zinc ion, and the water-insoluble nature sustained-release constituent that consists of an acid mucopolysaccharide object.
[Claim 2] The water-insoluble nature sustained-release constituent according to claim 1 characterized by the protein or the peptide which has said drug effect being G-CSF, IL-2, ETANASEPUTO, or an antibody.
[Claim 3] The water-insoluble nature sustained-release constituent according to claim 1 characterized by the content in the protein which has drug effect, or the water-insoluble nature sustained-release constituent of a peptide being at least 0.01 % of the weight.
[Claim 4] The water-insoluble nature sustained-release constituent according to claim 1 characterized by protein without said main drug effect being a human serum albumin or gamma globulin.
[Claim 5] The water-insoluble nature sustained-release constituent according to claim 1 characterized by the content in the water-insoluble nature sustained-release constituent of protein without said main drug effect being at least 1 % of the weight.
[Claim 6] The water-insoluble nature sustained-release constituent according to claim 1 characterized by the content in the water-insoluble nature sustained-release constituent of said zinc ion being at least 1 % of the weight.
[Claim 7] The water-insoluble nature sustained-release constituent according to claim 1 characterized by said acid mucopolysaccharide object being at least one of chondroitin sulfate, hyaluronic acid, heparin, a heparan sulfate, dermatan sulfate or keratan sulfates, and the salts of those.
[Claim 8] The water-insoluble nature sustained-release constituent of the protein content in which the content in the water-insoluble nature sustained-release constituent of said acid mucopolysaccharide object does not have main drug effect according to claim 1 characterized by a certain thing 1/100 or more.
[Claim 9] The water-insoluble nature sustained-release constituent characterized by freeze-drying a water-insoluble nature sustained-release constituent according to claim 1.
[Claim 10] A water-insoluble nature sustained-release constituent given in either of claim 1 to claims 9 characterized by being the gestalt to which said water-insoluble nature sustained-release constituent fitted subcutaneous injection, intradermal injection, and an intramuscular injection.
[Claim 11] The water-insoluble nature sustained release drug characterized by consisting of having added the additive which can be received in galenical pharmacy to the constituent according to claim 1 if needed.
[Claim 12] Pharmaceutical preparation according to claim 11 characterized by said additive which can be received in galenical pharmacy being a lactic-acid glycolic-acid polymer, a lactic-acid polymer, a surfactant, antiseptics, or a stabilizing agent.
[Claim 13] Pharmaceutical preparation characterized by freeze-drying pharmaceutical

preparation according to claim 11.
[Claim 14] Pharmaceutical preparation given in either of claim 11 to claims 13 characterized by being the gestalt to which said pharmaceutical preparation fitted subcutaneous injection, intradermal injection, and an intramuscular injection.
[Claim 15] The manufacture approach of the water-insoluble nature sustained-release constituent which mixes the protein which has the drug effect which forms zinc ion and precipitate or the solution of a peptide, the solution of zinc ion and protein without the main drug effect which forms precipitate, the solution of zinc salt, and the solution of an acid mucopolysaccharide object, and is produced.
[Claim 16] (1) The manufacture approach of the water-insoluble nature sustained-release constituent according to claim 1 which mixes the solution of the protein which has the drug effect which forms zinc ion and precipitate, or a peptide, and the solution of zinc salt, carries out precipitate creation, and is characterized by subsequently mixing (2) zinc ion, the solution of protein without the main drug effect which forms precipitate, and the solution of an acid mucopolysaccharide object.
[Claim 17] The manufacture approach of the water-insoluble nature sustained-release constituent according to claim 1 which considers the water-insoluble nature sustained-release constituent by the manufacture approach according to claim 15 or 16 as precipitate according to centrifugal separation, and is characterized by re-suspending the precipitate with a zinc salt solution [claim 18] It is 1,000 x g at least about the centrifugal separation of claim 17. The manufacture approach of the water-insoluble nature sustained-release constituent according to claim 1 characterized by carrying out.
[Claim 19] The manufacture approach of the water-insoluble nature sustained-release constituent according to claim 1 characterized by mixing the protein which has the drug effect which forms said zinc ion and precipitate or the solution of a peptide, the solution of zinc ion and protein without the main drug effect which forms precipitate, the solution of zinc salt, and the solution of an acid mucopolysaccharide object on condition that pH 4.5-8.5.

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DETAILED DESCRIPTION

[Detailed Description of the Invention]
[0001]
[Field of the Invention] The water-insoluble nature sustained-release constituent which carried out coprecipitation of the protein which does not have main drug effect although this invention forms the protein or the peptide, the zinc ion, and precipitate which have the drug effect which forms zinc ion and precipitate by addition of zinc salt to the bottom of acid mucopolysaccharide existence, its pharmaceutical preparation, and its manufacturing method, The protein or the peptides which form zinc ion and precipitate in detail, such as G-CSF, the protein which does not have main drug effect although zinc ion and precipitate are formed, an acid mucopolysaccharide object, the water-insoluble nature sustained-release constituent using the water-insoluble nature precipitate which consists of zinc ion, and its manufacturing method list -- it is related with the sustained release drug using it.
[0002]
[Description of the Prior Art] G-CSF pharmaceutical preparation is used to current, and the disease and symptom by which reduction in neutrophil leucocyte is accompanied. Although the medication method is an intravenous injection, subcutaneous injection, intravenous drip, etc., administration is that it is prescribed for the patient every day by 1 time per or 2 times day. This is because G-CSF more than a certain concentration needs to exist in blood in order to maintain drug effect to the top where the blood Nakayasu quality of G-CSF is bad and where a half-life is short. Therefore, the patient had forced a burden called administration every day, and has led also to extensive use of G-CSF. Therefore, pharmaceutical preparation-ization for maintaining the blood drug concentration of G-CSF is needed.
[0003] Some amino acid of current and G-CSF is permuted, and although the pharmaceutical preparation of the variant which extended the half-life is also used, the half-life has not resulted in the improvement of the count of administration in about 2 times. Moreover, although development of PEG-ized G-CSF which added the polyethylene glycol (PEG) to some of lysine residue of G-CSF is also made, if the fall of the activity by PEG-izing, antigenic, a production cost, etc. are taken into consideration, development of the blood-drug-concentration maintenance pharmaceutical preparation of still more desirable G-CSF is desired.
[0004] Although gradual release-ization of a drug is a good means to maintain long duration drug concentration in the living body, if stability is considered when using protein, not much excessive conditions cannot be used but will receive many limits in production of the gradual release pharmaceutical preparation.
[0005] It is very ******** about difficulty to look for the quiet conditions which the conditions which precipitate according to a proteinic class differ, and form precipitate, although there is a method of building and using precipitate on conditions quiet as easy proteinic gradual-release-izing. Furthermore, the condition must promise high yield. Although precipitate with isoelectric point precipitate, a salting-out, and a metal ion etc. can be applied and zinc suspension pharmaceutical preparation is actually used by clinical by the insulin as an approach of settling protein, the dissolution of precipitate does not make effectiveness maintain for several days early in the living body. Therefore, the gradual release pharmaceutical preparation which added

the device further, not using the protein made precipitate for maintaining drug effect several days as it is is needed.
[0006] Or although the effectiveness expected from the protein of the basicity which has gel and compatibility for acquiring the gradual release effectiveness in this case, or heparin affinity although the gradual release agent which uses hydro gel using gelatin, hyaluronic acid, etc. as a carrier is also considered is acquired, it is thought that the gradual release effectiveness that it is desirable for natural diffusion is not acquired even if the isoelectric point makes it involve in gel in the protein below neutrality like G-CSF.
[0007] Moreover, although there is an application for patent No. 177548 [2001 to] for which these people applied as advanced-technology reference on June 11, Heisei 13, there is no publication about G-CSF in this reference.
[0008]
[Problem(s) to be Solved by the Invention] Thus, though G-CSF is one of the cytokine by which clinical use is carried out most, the present condition is that the pharmaceutical preparation which maintains blood drug concentration for several days is not developed. Then, this invention is easy, and moreover, by the manufacture approach of high yield, it aims at offering the particle pharmaceutical preparation of the water-insoluble nature which holds drug effect for several days in the living body according to the gradual release effectiveness while stabilizing G-CSF by precipitate-ization.
[0009]
[Means for Solving the Problem] this invention persons tried development of the gradual release pharmaceutical preparation according [a granulocyte colony-stimulating factor (G-CSF)] to the precipitate nature particle paying attention to a metal ion, for example, calcium ion, and coming out and forming precipitate. As mentioned above, it was easy to dissolve precipitate of only protein and polyvalent metal ion, and it was expected that the gradual release effectiveness expected if it remains as it is was not acquired, and the experiment which this invention persons conducted was also actually checked.
[0010] Then, this invention persons tried making precipitate with the metal ion of G-CSF contain the protein which thinks whether it is effective to add the matter of precipitate nature to the constituent of precipitate further as an approach of suppressing the dissolution of such precipitate that consists of G-CSF and a metal ion, and combining a metal ion is known, and does not almost have drug effect, for example, a human serum albumin, gamma globulin, etc. In this case, it is important to choose the polyvalent metal ion which can precipitate both G-CSF and such protein. Since a human serum albumin and gamma globulin did not form precipitate in calcium ion, this invention persons actually investigated precipitate formation about each ion of calcium, zinc, copper, iron, aluminum, tin, and nickel on some polyvalent metal ion and a concrete target. As a result, with calcium, iron, aluminum, and nickel, precipitate was not caused at all but it turned out that zinc ion is the most desirable. In order zinc acetate or a zinc chloride is desirable and to gather the yield of precipitate as a salt to be used, zincky concentration was understood that 5 or more mMs are desirable. G-CSF was able to be settled 95% or more with the zinc ion of the concentration of 5 or more mMs.
[0011] Furthermore, by adding an acid mucopolysaccharide object in this invention, the above-mentioned mixed protein could be precipitated still more efficiently, and it found out that sustained-release improved further. Although there are chondroitin sulfate, hyaluronic acid, heparin, a heparan sulfate, dermatan sulfate, keratan sulfates, these salts, etc., chondroitin sulfate and its salt of the mucopolysaccaride are the most desirable.
[0012] For example, although it precipitated about 80 to 90% with the zinc chloride of 10mM(s) in 20 mg/ml gamma globulin, it checked that 99% precipitated by adding chondroitin sulfate. Moreover, it was able to be made to precipitate 95% or more in a 10mg [/ml] human serum albumin by adding chondroitin sulfate in the zinc chloride of 20mM(s), although precipitate is about 10%.
[0013] In addition, although adding serum proteins if needed to protein or a peptide etc. which has biological activity is indicated by said reference (application for patent No. 177548 [2001 to]), it is indispensable to produce the precipitate to which making zinc ion and protein without

the main drug effect which forms precipitate contain surely raised the gradual release effectiveness to precipitate of G-CSF, the below-mentioned IL-2, ETANASEPUTO or an antibody, and zinc ion like the above-mentioned in this invention.
[0014] Thus, this invention persons resulted [that gamma globulin a human serum albumin, etc. and an acid mucopolysaccharide object are added further precipitate formation is promoted by those interactions in the approach of making new precipitate forming, and G-CSF can be settled 99% or more in a solution including precipitate by the zinc ion of G-CSF, and] in a header and this invention. This precipitate is sufficient fineness to pass along a hypodermic needle, and is usable as injections.
[0015] Furthermore, it was checked in the experiment using a mouse that the gradual release effectiveness in the living body also increases very much, and gamma globulin and the effectiveness of human serum albumin precipitate formation of having added the acid mucopolysaccharide object not only raise the yield of the precipitate formation by the zinc ion of G-CSF, but completed this invention.
[0016] This invention is characterized by making precipitate form between pH 4.5-8.5. That is, since it is pH near neutrality, it can manufacture, without spoiling the activity also in protein which loses activity by extreme pH like G-CSF. Furthermore, it is characterized by including G-CSF in precipitate at a high rate. On the optimal conditions, it is 99% or more.
[0017] Although it is possible to also make precipitate form without caring about the sequence of adding each constituent as for the water-insoluble nature sustained-release constituent of this invention, in order to include G-CSF in precipitate by high yield, it is desirable for a 10-1000microg [/ml] G-CSF solution to settle G-CSF first with the zinc acetate or the zinc chloride of the last concentration of 5 or more mMs. Next, it is desirable to make the water-insoluble nature sustained-release constituent of this invention from adding chondroitin sulfate so that the last concentration may be set to 0.5 - 20 mg/ml and it may become the last concentration of 1 / 2 - 1/100 to a human serum albumin and albumin.
[0018] Furthermore, after hardening precipitate by performing centrifugal actuation, in the water solution containing zinc ion, it re-suspends and can also use. Furthermore, with water for injection, what added the mannitol etc. and was freeze-dried is re-suspended in suspension, and can be used for it. Precipitate was melted to EDTA, electrophoresis of the conditions of not denaturalizing was performed, and it was checked that G-CSF has not caused denaturation during precipitate creation. Moreover, with the freeze-dried pharmaceutical preparation, it was checked at 37 degrees C that one-month or more G-CSF has not denaturalized. The G-CSF pharmaceutical preparation of this invention can be prescribed for the patient by subcutaneous injection or the intramuscular injection. In order that not only maintaining the drug effect ten days or more by one administration but its dose might maintain the drug effect in a mouse, 1/10 or less [of the amount of G-CSF used by administration every day] was enough as the G-CSF pharmaceutical preparation of this invention. Moreover, the G-CSF pharmaceutical preparation of this invention maintained the drug effect the 6th day or more by one administration in the rat, and in order to maintain the drug effect, 1/3 or less [of the amount of G-CSF used by administration every day] was enough as the dose at that time.
[0019] The gradual release pharmaceutical preparation by the manufacture approach of this invention is applicable to protein or a peptide with the drug effect which makes not only G-CSF but zinc ion, and precipitate. For example, the recombination object protein preparation which attached hirudine, interleukin-2 (IL-2), ETANASEPUTO, a monoclonal antibody, protein with gamma-carboxyglutamic acid, and a His-tag is contained. The factor which shows drug effect especially in a minute amount is desirable.
[0020] For example, that which formed the monoclonal antibody into gradual release pharmaceutical preparation by the approach of this invention showed the same gradual release effectiveness as G-CSF in the experiment of in vitro. Moreover, although IL-2 precipitated with the zinc chloride, it was checked that the way in which added a human serum albumin and chondroitin sulfate and precipitate was made to form is included more in precipitate.
[0021]
[Example] The example of this invention is explained below.

(Example 1 of a trial) 20micro (chlorination tin, an aluminum chloride, a copper chloride, ferric chloride, a zinc chloride, a calcium chloride, nickel chloride) of 500 metal salts l of mM(s) was added to 500micro of attempt 150microg of precipitate creation with various metal ions of G-CSF/ml G-CSF (Chugai Pharmaceutical) solutions l, it put after churning, and precipitate formation was checked by viewing. Precipitate was formed to all six kinds of metal salts.

[0022] (Example 2 of a trial) 20micro (chlorination tin, an aluminum chloride, a copper chloride, ferric chloride, a zinc chloride, a calcium chloride, nickel chloride) of 500 metal salts l of mM(s) was added to the 10mg [/ml] effectiveness human gamma globulin (sigma company) or 500micro of 10mg [/ml] human serum albumin (sigma company) solutions l which human gamma globulin, and the attempt and mucopolysaccaride of various metal ion formation of a human serum albumin give to precipitate formation, it put after churning, and precipitate formation was checked by viewing. Both protein became cloudy by addition of chlorination tin and a zinc chloride. When 20 moremg [/ml] chondroitin sulfate was added in these solutions, precipitate was checked with all solutions other than a calcium chloride addition solution. About chlorination tin and a zinc chloride, it became cloudy still more strongly. Moreover, precipitate of an aluminum chloride, ferric chloride, and a nickel chloride addition solution was not uniform precipitate about an aluminum chloride and ferric chloride weakly. It turned out that zinc is the optimal as a metal ion which settles gamma globulin and a human serum albumin from a result.

[0023] 0.5M zinc chloride (pH5.4), respectively to 500micro [of gamma globulin of 20mg/ml of precipitate creation by the zinc chloride and chondroitin sulfate of gamma globulin] (sigma company) l 20, (Example 3 of a trial) 10, 5, 2, and the thing that added 10micro of 0.5M zinc chlorides l and 160micro [of 20mg //ml / chondroitin sulfate] l to what was added, and 500micro [of 1 or 0.5micro l (20-0.5mM) 20mg //ml / gamma globulin] l were left in the room temperature for after [churning] 5 minutes. Next, at-long-intervals alignment separation was carried out by about 10,000 x g for 10 minutes, and the gamma globulin concentration of supernatant liquid was measured by the coloring method using a Protein Assay reagent (Bio-Rad).

[0024] The amount of precipitate of gamma globulin increases as are shown in Table 1, and zinc chloride concentration becomes high. Moreover, chondroitin sulfate is promoting precipitate formation.

表１γ-グロブリンと亜鉛イオンの沈殿形成における亜鉛塩の濃度の影響とコンドロイチン硫酸（CS）の効果

塩化亜鉛濃度 (mM)	0.5	1	2	5	10	20	10 +CS
上清に残るγ-グロブリンの割合 (%)	39	37	37	30	13	5	2

[0025] (Example 4 of a trial) the solution which mixed milli Q water or 200micro [of 10mg //ml / hyaluronic acid] l at 300micro [of precipitate 20mg //ml / creation gamma globulin] l by the zinc chloride and hyaluronic acid of gamma globulin, and was set to 500microl -- a 0.5M zinc chloride (pH5.4) -- 20, 10, and 5 -- 2 or 1microl (20-1mM) in addition, it was left for 5 minutes in the room temperature after churning. Next, the gamma globulin concentration of supernatant liquid was measured by the coloring method using a Protein Assay reagent (Bio-Rad) by about 10,000 xg(s) after 4 degrees C and 30-minute alignment separation at long intervals. As shown in Table 2, hyaluronic acid as well as chondroitin sulfate promoted precipitate formation of the gamma globulin by the zinc chloride.

表2 γ-グロブリンと亜鉛イオンの沈殿形成における亜鉛塩の濃度の影響とコンドロイチン硫酸（CS）の効果

亜鉛濃度（mM）	ヒアルロン酸なし					ヒアルロン酸添加				
	1	2	5	10	20	1	2	5	10	20
上清に残るγ-グロブリンの割合（%）	70	76	47	15	5	41	11	4	3	3

[0026] 20mg [/ml] chondroitin sulfate (Wako Pure Chem) to gamma globulin and 200micro [of 20mg //ml / ratio-of-concentration gamma globulin of chondroitin sulfate] (sigma company) I in gamma globulin, zinc, and chondroitin sulfate precipitate 100microl (2:1), (Example 5 of a trial) 50microl (4:1), 25microl (8:1), 12.5microl (16:1), or 10mg [/ml] chondroitin sulfate 40microl (10:1), 20microl (20:1), 8microl (50:1), 4microl (100:1), or 1mg [/ml] chondroitin sulfate 8microl (500:1), 4microl (1000:1) was mixed, 0.5M zinc chloride (pH5.4) was 8microl Added to the solution set to 400microl with milli Q water, and it was left for 5 minutes at the room temperature. Next, at-long-intervals alignment separation was carried out at about 10,000 xg(s) and 4 degrees C for 10 minutes, and the gamma globulin concentration of supernatant liquid was measured by the coloring method using a Protein Assay reagent (Bio-Rad). In 90% or more of the result, the ratio of gamma globulin and chondroitin sulfate had precipitated in 2-100:1. Moreover, the time of ratios being 8-20:1 had precipitated most.

[0027] (Example 6 of a trial) in addition, it was referred to as 500microl with milli Q water, and was left for 10 minutes at 37 degrees C after churning so that 0.5M zinc acetate (pH6.0) might be used for 100micro [of precipitate creation 50 mg/ml human serum albumins] (sigma company) I of the zinc acetate of a human serum albumin, and chondroitin sulfate and zinc concentration might serve as 1, 5, 10, 15, and 20mM. Since it became cloudy, at-long-intervals alignment separation was carried out by about 10,000 xg(s) for room temperature 10 minutes, and the concentration of the human serum albumin of supernatant liquid was measured by the coloring method using a Protein Assay reagent (Bio-Rad). The percentage of the human serum albumin which precipitated by all zinc concentration was only 2 - 10%. Next, 50mg [/ml] human serum albumin (sigma company) 100microl It adds so that zinc concentration may serve as 1, 5, 10, 20, and 40mM using 0.5M zinc acetate (pH6.0). It prepared in the solution of 500microl using 20mg [/ml] chondroitin sulfate and Miri Q, and was left for 10 minutes at 37 degrees C after churning so that the concentration of chondroitin sulfate might become [ml] by each zinc concentration in 0.5, 1 and 2.5, and 5 or 10mg /. Since precipitate was formed, the albumin concentration of supernatant liquid was measured by the coloring method using a Protein Assay reagent (Bio-Rad) after 10-minute alignment separation at long intervals at about 10,000 xg room temperature. As shown in Table 3, 1mg [ml] /, i.e., human serum albumin:chondroitin sulfate, has 20mM(s) and the best amount of chondroitin sulfate by the concentration of 10:1, and zinc concentration precipitated.

表3 10mg/mlのヒト血清アルブミン（HSA）の沈殿量に与える亜鉛塩とコンドロイチン硫酸の濃度の影響

		亜鉛濃度 （mM）				
		1	5	10	20	40
コンドロイチン硫酸濃度（mg/mL）		上清に残るHSAの割合（%）				
	0.5	83	15	4	1	5
	1	80	29	2	1	1
	2.5	85	56	14	3	1
	5	77	84	37	9	3
	10	78	84	71	27	7

[0028] (Example 1) 103micro of elution test G-CSF(150microg/(ml)) 20microl in the creation of precipitate gradual release pharmaceutical preparation and in vitro of G-CSF by the human serum albumin of G-CSF, zinc acetate, and chondroitin sulfate mixture, zinc acetate (0.5M) 12microl, and milli Q water I was made to mix and suspend, human serum albumin (20mg/(ml)) 150microl and 15micro [of chondroitin sulfate] (20mg/(ml)) I were added further, and it put for 10 minutes at 37 degrees C after churning. After a 10-minute alignment at long intervals and supernatant liquid were re-suspended at about 10,000 xg(s) and a room temperature by ****, 20mM zinc acetate of 150microl, 0.5M carboxymethyl cellulose, and 5% mannitol, and it considered as gradual release pharmaceutical preparation. Moreover, control used 20microl G-CSF (150microl./(ml)) as 150microl solution with milli Q water. The elution test of G-CSF from precipitate was performed using the 24 well-cel culture insertion system (falcon company). After paying the sample of 100microl to the insertion with the film with a pore size of 3 micrometers, the insertion was put into the well of 900microl into which BSA and PBS went 0.3%, and it swayed with the shaker. The insertion was moved and changed into a new well for every hour, the quantum of G-CSF eluted in the well through the film of an insertion was carried out by ELISA, and the elution volume was integrated for every hour till 8 hours. As shown in drawing 1, it was shown that the addition elution volume from precipitate 8 hours after has the gradual release effectiveness considerably at about 5%.

[0029] (Example 2) after adding the 0.5M zinc acetate of 20microl, and the 20mg [/ml] chondroitin sulfate of 25microl to 150microg [the moving state in blood of G-CSF in a G-CSF gradual release pharmaceutical preparation administration normal mouse, and] of 167micro of drug effect I/ml G-CSF (Chugai Pharmaceutical), 100micro [of 50mg //ml / human serum albumins] I and 188micro of water for injection I were added and agitated, and it was left for 5 minutes at 37 degrees C. Supernatant liquid was thrown away after 10-minute alignment separation at long intervals by about 10,000 xg(s), and precipitate was suspended in 20mM zinc acetate of 250microl, and 5% mannitol solution of 0.5% carboxymethyl-cellulose content. The intramuscular injection of the 100micro of this suspension I was carried out to three normal mice (C3H) with a weight of about 25g. 100micro [of 50mg //ml / human serum albumins] I and 117micro of water for injection I were added to 150microg of 33microl/ml G-CSF as control, and the intramuscular injection of the 100microl of the solution made into the mannitol 5% was carried out to three normal mice (C3H) with a weight of about 25g. Collecting blood before administration on 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, and the 14th [after / administration / 4 hours, and], the moving state in blood of G-CSF was measured by ELISA till the 6th, and measured the white blood cell count in the erythrocytometer as drug effect till the 14th. Although what prescribed G-CSF itself for the patient existed in blood considerably after 4 hours as shown in drawing 2, 24 hours after was below limit of detection. Compared with it, the direction of gradual

release pharmaceutical preparation existed in blood by several 100 pg(s)/ml concentration six days after. The effectiveness of making a leucocyte increasing for 11 days about drug effect was maintained.

[0030] (Example 3) Creation, its drug effect and stability G-CSF(150microg/(ml)) 1.334ml of freeze-drying G-CSF gradual release pharmaceutical preparation, 0.8ml (0.5M) of zinc acetate, and 12.866ml of water for injection were mixed, after churning, 1ml of 20mg [/ml] chondroitin sulfate and 4ml of 50mg [/ml] human serum albumins were added, they were agitated, and it was left at 37 degrees C for 10 minutes. Supernatant liquid was re-suspended by **** and 18ml 20mM zinc acetate after the 10-minute alignment at long intervals in about 10,000 xg(s), and the 1g mannitol was added and agitated. 1.2ml poured distributively and freeze-dried at a time to the vial, agitating. Subcutaneous injection of the 300micro of the solutions I which made the lyophilized products set at 37 degrees C for two weeks suspend by the carboxymethyl cellulose or 1ml of water for injection 0.5% was carried out to about 25g mouse. It collected blood before administration on 1, 2, 3, 4, 5, 7, 8, 9, 10, and the 11th after administration, and the white blood cell count was measured in the erythrocytometer. As shown in drawing 3, in both lyophilized products, drug effect was observed for ten days. G-CSF freeze-dried to the vial was kept for one month at 37 degrees C, precipitate was melted by 1ml EDTA (100mM), native electrophoresis of the 10microl was carried out using the multi-gels 2/15 (the 1st chemistry), and the denaturation of G-CSF, decomposition, condensation, etc. were investigated by detecting G-CSF by the argentation and the after [waste turn BUROTINGU] anti-G-CSF antibody. Consequently, the mobility in the electrophoresis of G-CSF of pharmaceutical preparation did not change at all, and a band which originates in decomposition or condensation was not accepted. The result showed that G-CSF lyophilized products were very stable.

[0031] (Example 4) after adding the 0.5M zinc acetate of 20microl, and the 20 mg/ml chondroitin sulfate of 25microl to 780microg of 32micro of drug effect I of G-CSF in G-CSF gradual release pharmaceutical preparation administration normal rat/ml G-CSF (Chugai Pharmaceutical), 100microl and 323micro of water for injection I were added, the 50 mg/ml human serum albumin was agitated, and it was left for 5 minutes at 37 degrees C. Supernatant liquid was thrown away after the 10-minute alignment at long intervals by about 10,000 xg(s), and precipitate was suspended in 20mM zinc acetate of 500microl, and 5% mannitol solution of 0.5% carboxymethyl-cellulose content. Subcutaneous injection of the 200micro of this suspension I was carried out to two normal rats (Wister) with a weight of about 120g. It carried out subcutaneous injection of 25microg [a single time and]/ml G-CSF and the 200micro of the 5% mannitol liquid I of 10 mg/ml human serum albumin content at a time for the precipitate suspension (vehicle) which does not contain G-CSF as contrast to two normal rats (Wister) with a weight of about 120g every day on the 5th. It collected blood before administration on 1, 2, 3, 4, 6, 7, and the 8th after administration, and the white blood cell count was measured in the erythrocytometer as drug effect till the 8th. Although the white blood cell count returned to the basis when the thing of every day administration of contrast stopped administration, as shown in drawing 4 , gradual release pharmaceutical preparation was maintaining the effectiveness of making a leucocyte increasing for six days by one administration.

[0032] (Example 5) 180micro of elution anti-TNF antibody (10mg/(ml)) 25microl in in vitro of the zinc ion of an anti-TNF antibody and the anti-TNF antibody from human serum albumin chondroitin sulfate mixing precipitate, zinc chloride (0.5M) 20microl, and milli Q water I was mixed, and it stirred, human serum albumin (20mg/(ml)) 250microl and 25micro [of chondroitin sulfate] (20mg/(ml)) I were added and stirred further, and it put at 37 degrees C for 10 minutes. After 10-minute alignment separation at long intervals and supernatant liquid were re-suspended at about 10,000 xg(s) and a room temperature by 20mM zinc chloride content 0.5M carboxymethyl cellulose of **** and 500microl, and it considered as gradual release pharmaceutical preparation. moreover, control -- 10microl -- it considered as the solution which added anti-TNF (10mg/(ml)) and 180microl human serum albumin (20mg/(ml)). The elution test of the anti-TNF antibody from precipitate was performed using the 24 well-cel culture insertion system (falcon company). After paying the sample of 100microl to the insertion with the film with a pore size of 3 micrometers, the insertion was put into the well of 900microl into which BSA

and PBS went 0.3%, and it swayed with the shaker. The insertion was moved and changed into a new well for every hour, the quantum of the anti-TNF antibody which has taken out to the well through the film of an insertion was carried out by ELISA, and the elution volume was integrated for every hour till 8 hours. As shown in drawing 5 , it was shown that the elution from precipitate 8 hours after has the gradual release effectiveness considerably at 10% or less.

[0033] (Example 6) 46micro of formation IL-2(100microg/(ml)) 50microl, zinc chloride (0.5M) 4microl, and milli Q water l of precipitate containing the precipitate formation and the human serum albumin by the zinc chloride, and chondroitin sulfate of IL-2 was mixed and stirred, and it was left in the room temperature for 10 minutes. Recovery and precipitate melted supernatant liquid by 50mM EDTA of 100microl by about 10,000 xg(s) after room temperature 10-minute alignment separation at long intervals. Moreover, IL-2(100microg/(ml)) 50microl and 4micro (0.5M) of zinc chlorides l were mixed, and after stirring, 21micro of chondroitin sulfate (20mg/(ml)) 5microl, human serum albumin (50mg/(ml)) 20microl, and milli Q water l was mixed, and it stirred again, and was left for 10 minutes. Recovery and precipitate melted supernatant liquid by 50mMEDTA(s) of 100microl after the 10-minute alignment at long intervals by about 10,000 xg (s). 10micro of liquid l which melted these supernatant liquid and precipitate was mixed with the electrophoresis sample processing liquid of 10microl, and SDS electrophoresis was performed using the multi-gels 10/20 (the 1st chemistry). Coomassie brilliant blue staining was performed and IL-2 were detected. In which conditions, IL-2 were detected in the direction of precipitate and they were not detected by the result in the direction of supernatant liquid. This shows that the formation of gradual release pharmaceutical preparation is possible by the approach as G-CSF that IL-2 are the same.

[Translation done.]

* NOTICES *

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2.**** shows the word which can not be translated.
3.In the drawings, any words are not translated.

DESCRIPTION OF DRAWINGS

[Brief Description of the Drawings]
[Drawing 1] It is drawing having shown the elution of G-CSF compared with the solution of G-CSF in the experiment of in vitro from the pharmaceutical preparation of an example 7.
[Drawing 2] It is drawing showing transition of the blood drug concentration of G-CSF after an intramuscular injection, and change of the white blood cell count of a mouse for the pharmaceutical preparation of an example 8 to a mouse.
[Drawing 3] When the lyophilized products of an example 9 are administered hypodermically to a mouse, it is drawing showing that drug effect is maintained by gradual release of G-CSF.
[Drawing 4] It is drawing in which injecting hypodermically [of a rat] with the pharmaceutical preparation of an example 10, and showing the comparison of drug effect with every day administration of a G-CSF solution as contrast.
[Drawing 5] It is drawing having shown the elution of an anti-TNF antibody compared with the solution of an anti-TNF antibody in the experiment of in vitro from the pharmaceutical preparation of an example 7.

[Translation done.]

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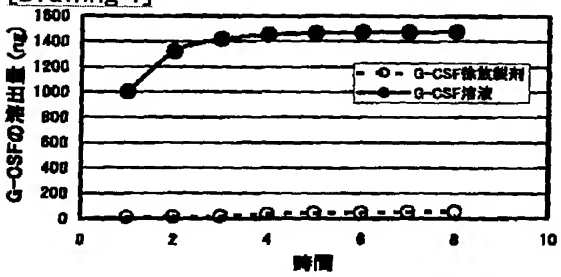
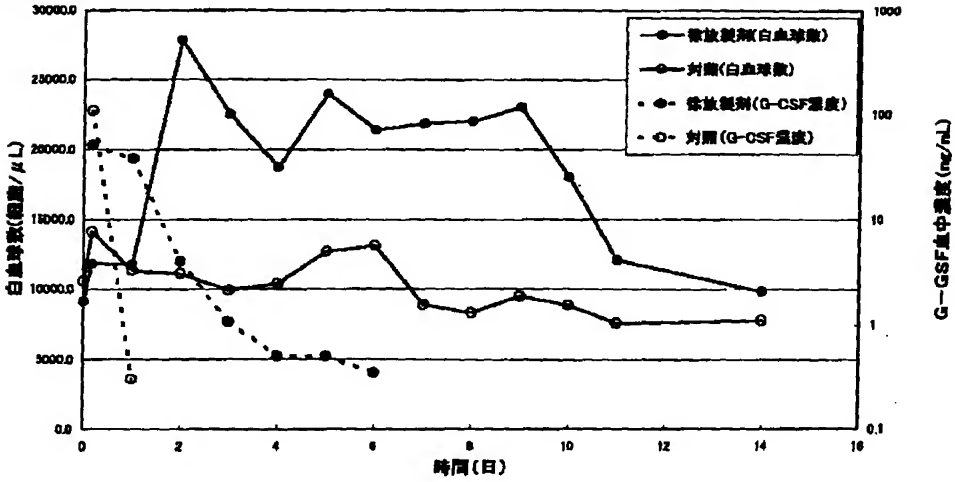
DRAWINGS

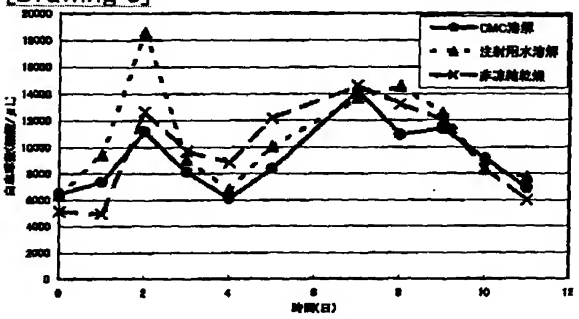
[Drawing 1]



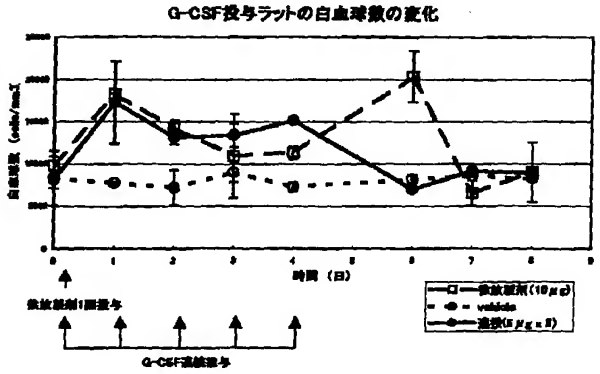
[Drawing 2]



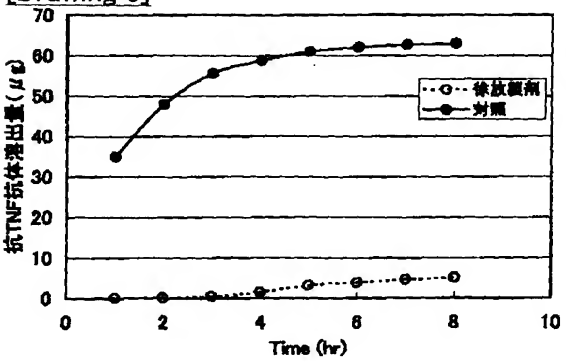
[Drawing 3]



[Drawing 4]



[Drawing 5]



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